COMBINATION OF AD-P53 AND CHEMOTHERAPY FOR THE TREATMENT OF TUMOURS

I. Field of the Invention

The present invention relates generally to the fields of oncology, pathology, molecular biology and gene therapy. More particularly, it concerns the use of p53 gene therapy to provide clinical benefit in patients with recurrent cancer treated with radiation and/or chemotherapy.

II. Description of Related Art

Cancer is a leading cause of death in most countries, and the result of billions of dollars in healthcare expense around the world. Through great effort, significant advances have been made in treating cancer, primarily due to the development of radiation and chemotherapy-based treatments. Unfortunately, a common problem is tumor cell resistance to radiation and chemotherapeutic drugs. For example, NSCLC accounts for at least 80% of the cases of lung cancer, but patients with NSCLC are generally unresponsive to chemotherapy (Doyle, 1993). One goal of current cancer research is to find ways to improve the efficacy of these "traditional" therapeutic regimens, and the genetics of cancer cells has led to dramatic discoveries and a greater understanding of disease development.

It is now well established that a variety of cancers are caused, at least in part, by genetic abnormalities that result in either the overexpression of cancer causing genes, called "oncogenes," or from loss of function mutation in protective genes, often called "tumor suppressor" genes. An important gene of the latter category is p53 - a 53 kD nuclear phosphoprotein that controls cell proliferation. Mutations to the p53 gene and allele loss on chromosome 17p, where this gene is located, are among the most frequent alterations identified in human malignancies. The p53 protein is highly conserved through evolution and is expressed in most normal tissues. Wild-type p53 has been shown to be involved in control of the cell cycle (Mercer, 1992), transcriptional regulation (Fields and Jang, 1990; Mietz *et al.*, 1992), DNA replication (Wilcock and Lane, 1991; Bargonetti *et al.*, 1991), and induction of apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992).

Various mutant p53 alleles are known in which a single base substitution results in the synthesis of proteins that have quite different growth regulatory properties and, ultimately, lead to malignancies (Hollstein *et al.*, 1991). In fact, the p53 gene has been found to be the most frequently mutated gene in common human cancers (Hollstein *et al.*, 1991; Weinberg, 1991), and is particularly associated with those cancers linked to cigarette smoke (Hollstein *et al.*, 1991; Zakut-Houri *et al.*, 1985). The overexpression of p53 in breast tumors has also been documented (Casey *et al.*, 1991). Interestingly, however, the beneficial effect of p53 are not limited to cancers that contain mutated p53 molecules. In a series of papers, Clayman *et al.* (1994; 1995a; 1995b) demonstrated that growth of cancer cells expressing wild-type p53 molecules was nonetheless inhibited by expression of p53 from a viral vector.

As a result of these findings, considerable effort has been placed into p53 gene therapy. Retroviral delivery of p53 to humans was reported some time ago (Roth *et al.*, 1996). There, a retroviral vector containing the wild-type p53 gene under control of a beta-actin promoter was used to mediate transfer of wild-type p53 into 9 human patients with non-small cell lung cancers by direct injection. No clinically significant vector-related toxic effects were noted up to five months after treatment. *In situ* hybridization and DNA polymerase chain reaction showed vector-p53 sequences in post-treatment biopsies. Apoptosis (programmed cell death) was more frequent in post-treatment biopsies than in pretreatment biopsies. Tumor regression was noted in three patients, and tumor growth stabilized in three other patients. Similar studies have been conducted using adenovirus to deliver p53 to human patients with squamous cell carcinoma of the head and neck (SCCHN) (Clayman *et al.*, 1998). Surgical and gene transfer-related morbidities were minimal, and the overall results provided preliminary support for the use of Ad-p53 gene transfer as a surgical adjuvant in patients with advanced SCCHN.

Despite these successes, there remains a need to identify specific patient subsets that will most benefit from these procedures, and as a corallary, to identify methods which improve the chance of clinical benefit to these patients.

SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided a method of treating a subject with recurrent cancer comprising (a) selecting a patient based on (i) prior treatment of cancer with surgery, or a radio- or chemotherapy; and (ii) recurrence of cancer subsequent to said treatment, and (b) administering to said subject an expression construct comprising a nucleic acid segment encoding p53, said segment under the control of a promoter active in a cancer cell of said subject, said expression construct expressing p53 in said cancer cell. A subsequent step (c) that follows step (b) of administering to said subject a second radio- or chemotherapy, whereby said expression construct sensitizes said cancer cell to said second radio- or chemotherapy, thereby treating said cancer may also be provided.

The first radio- or chemotherapy and said second radio- or chemotherapy may be the same or different. The subject may be a non-human animal, or a human subject. The first and/or second radio- or chemotherapy may be chemotherapy, such as busulfan, chlorambucil, cisplatinum, cyclophosphamide, dacarbazine, ifosfamide, mechlorethamine, melphalan, 5-FU, Ara-C, fludarabine, gemcitabine, methotrexate, doxorubicin, bleomycin, dactinomycin, daunorubicin, idarubicin, mitomycin C, docetaxel, taxol, etoposide, paclitaxel, vinblastine, vincristine, vinorelbine, camptothecin, carmustine, or lomustine. The first and/or second radio- or chemotherapy may be radiotherapy, such as x-rays, gamma rays, or microwaves. The first and/or second radio- or chemotherapy may be characterized as a DNA damaging therapy.

The treated cancer may be brain cancer, head & neck cancer, esophageal cancer, tracheal cancer, lung cancer, liver cancer stomach cancer, colon cancer, pancreatic cancer, breast cancer, cervical cancer, uterine cancer, bladder cancer, prostate cancer, testicular cancer, skin cancer, rectal cancer lymphoma or leukemia.

The expression construct may be a viral expression construct, such as a retroviral construct, a herpesviral construct, an adenoviral construct, an adeno-associated viral construct, or a vaccinia viral construct. The viral expression construct may be a replication-competent virus or adenovirus, or a replication-defective virus or adenovirus. Alternatively, the expression construct may be a non-viral expression construct, such as

one that is comprised within a lipid vehicle. The promoter may be CMV IE, RSV LTR, β-actin, Ad-E1, Ad-E2 or Ad-MLP. Other gene therapy vectors and promoters known to those skilled in the art may also be utilized.

The time period between steps (b) and (c) may be about 24 hours, about 2 days, about 3 days, about 7 days, about 14 days, about 1 month, about 2 months, about 3 months, or about 6 months. Recurrence may be recurrence at a primary tumor site or a metastatic site. The subject may have had surgical resection prior to step (b), and/or the method may further comprise surgical resection following step (c). Administering in step (b) may be intratumoral, to a tumor vasculature, local to a tumor, regional to a tumor, or systemic. Administering in step (c) may be intratumoral, to a tumor vasculature, local to a tumor, regional to a tumor, or systemic.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

The term "about" means, in general, the stated value plus or minus 5%.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 – Advexin® Phase 2 Head and Neck Data on Recurrent or Refractory Disease (T201, T202 and T207 Lesional Response).

FIG. 2 – Advexin® Phase 2 Head and Neck Data (T201 versus T202; Increased Survival).

FIG. 3 – Advexin® Phase 2 Head and Neck Data Disease (T201+T202 vesrus T207; Increased Survival).

FIG. 4 – Advexin® Phase 2 Head and Neck Data (Combined, T201, T202 and T207 – Advexin® + Chemotherapy).

DESCRIPTION OF THE ILLUSTRATIVE EMBODIMENTS

I. The Present Invention

As discussed above, p53 gene therapy at the clinical level has been under study for a decade. Overall, the success of this approach has been remarkable, showing substantial increased benefits over than seen with traditional therapeutic approaches. Moreover, the side effects of gene therapy appear minimal, and there have been no confirmed deaths associated with the therapy. However, as with most anti-cancer treatments, there still remains a substantial need to improve the efficacy of p53 gene therapy.

In a retrospective analysis of Ad-p53 clinical trials, some remarkable observations have been made. While gene therapy alone provided substantial benefit to patients who exhibited recurrent cancer, patients receive a subsequent regimen of chemotherapy showed a dramatic increase in survival. Since patients that received the gene therapy had received at least one previous round of radio- or chemotherapy, the responsiveness of the cancer to a subsequent conventional treatment was quite unexpected.

Thus, the present invention focuses on treatment of a specific subset of patients – those with recurrent cancer. Such patients are those in the greatest need of new therapies, and recurrence of a primary cancer is a grave clinical indicator. In addition, the present invention provides an improved therapeutic regimen for these patients involving (a) prior therapy (surgery, radiation, chemotherapy or any combination thereof); (b) followed by p53 gene therapy. Further benefit can also be obtained by subsequent treatment with (c) at least one round of radio- or chemotherapy. Together, this particular treatment combination, on this particular patient subset, provides increased clinical benefits. While not entirely clear, the p53 may be providing a radiosensitizing or chemosensitizing effect to the recurrent tumors cells. Alternatively, the effect may derive from a partial or contributory apoptotis effect that is augmented by the radiation or chemotherapeutic.

The radio- or chemotherapy that is provided subsequent to p53 gene therapy may occur relatively quickly, although long enough after the p53 gene therapy to permit p53 expression. Thus, it is contemplated that earlier time points for subsequent therapy include as early as about 24 hours post-p53 treatment, but may range up to a 3- to 6-month time frame. The present invention may be utilized in a variety of cancers,

including sarcomas and carcinomas, and in particular, lymphomas, leukemias, gliomas, adenocarcinomas, squamous cell carcinomas (including head and neck), non-small cell cancer (including lung), melanomas, and others.

Delivery of the p53 expression constructs and/or chemotherapeutic drugs and/or radiation to patients is contemplated through a variety of different routes, using a variety of different regimens, and include local (intratumoral, tumor vasculature), regional and systemic delivery. Regimens for delivery of p53 gene therapy may follow those described in the examples, but more generally will involve one, two, three, four, five, six or more administrations of the p53 expression vector. Similarly, radio- or chemotherapy may be provided in multiple administrations.

The details for practicing the present invention are provided in the following pages.

II. p53

p53 is phosphoprotein of about 390 amino acids which can be subdivided into four domains: (i) a highly charged acidic region of about 75-80 residues, (ii) a hydrophobic proline-rich domain (position 80 to 150), (iii) a central region (from 150 to about 300), and (iv) a highly basic C-terminal region. The sequence of p53 is well conserved in vertebrate species, but there have been no proteins homologous to p53 identified in lower eucaryotic organisms. Comparisons of the amino acid sequence of human, African green monkey, golden hamster, rat, chicken, mouse, rainbow trout and *Xenopus laevis* p53 proteins indicated five blocks of highly conserved regions, which coincide with the mutation clusters found in p53 in human cancers evolution.

p53 is located in the nucleus of cells and is very labile. Agents which damage DNA induce p53 to become very stable by a post-translational mechanism, allowing its concentration in the nucleus to increase dramatically. p53 suppresses progression through the cell cycle in response to DNA damage, thereby allowing DNA repair to occur before replicating the genome. Hence, p53 prevents the transmission of damaged genetic information from one cell generation to the next initiates apoptosis if the damage to the cell is severe. Mediators of this effect included Bax, a well-known "inducer of apoptosis."

As discussed above, ,mutations in p53 can cause cells to become oncogenically transformed, and transfection studies have shown that p53 acts as a potent transdominant tumor suppressor, able to restore some level of normal growth to cancerous cells *in vitro*. p53 is a potent transcription factor and once activated, it represses transcription of one set of genes, several of which are involved in stimulating cell growth, while stimulating expression of other genes involved in cell cycle control

III. p53 Polynucleotides

Certain embodiments of the present invention concern nucleic acids encoding a p53. In certain aspects, both wild-type and mutant versions of these sequences will be employed. The term "nucleic acid" is well known in the art. A "nucleic acid" as used herein will generally refer to a molecule (*i.e.*, a strand) of DNA, RNA or a derivative or analog thereof, comprising a nucleotide base. A nucleotide base includes, for example, a naturally occurring purine or pyrimidine base found in DNA (*e.g.*, an adenine "A," a guanine "G," a thymine "T" or a cytosine "C") or RNA (*e.g.*, an A, a G, an uracil "U" or a C). The term "nucleic acid" encompass the terms "oligonucleotide" and "polynucleotide," each as a subgenus of the term "nucleic acid." The term "oligonucleotide" refers to a molecule of between about 8 and about 100 nucleotide bases in length. The term "polynucleotide" refers to at least one molecule of greater than about 100 nucleotide bases in length.

In certain embodiments, a "gene" refers to a nucleic acid that is transcribed. In certain aspects, the gene includes regulatory sequences involved in transcription or message production. In particular embodiments, a gene comprises transcribed sequences that encode for a protein, polypeptide or peptide. As will be understood by those in the art, this functional term "gene" includes genomic sequences, RNA or cDNA sequences or smaller engineered nucleic acid segments, including nucleic acid segments of a non-transcribed part of a gene, including but not limited to the non-transcribed promoter or enhancer regions of a gene. Smaller engineered nucleic acid segments may express, or may be adapted to express proteins, polypeptides, polypeptide domains, peptides, fusion proteins, mutant polypeptides and/or the like.

"Isolated substantially away from other coding sequences" means that the gene of interest forms part of the coding region of the nucleic acid segment, and that the segment does not contain large portions of naturally-occurring coding nucleic acid, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the nucleic acid as originally isolated, and does not exclude genes or coding regions later added to the nucleic acid by the hand of man.

A. Preparation of Nucleic Acids

A nucleic acid may be made by any technique known to one of ordinary skill in the art, such as for example, chemical synthesis, enzymatic production or biological production. Non-limiting examples of a synthetic nucleic acid (*e.g.*, a synthetic oligonucleotide), include a nucleic acid made by *in vitro* chemical synthesis using phosphotriester, phosphite or phosphoramidite chemistry and solid phase techniques such as described in EP 266 032, incorporated herein by reference, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.* (1986) and U.S. Patent 5,705,629, each incorporated herein by reference. Various mechanisms of oligonucleotide synthesis may be used, such as those methods disclosed in, U.S. Patents 4,659,774; 4,816,571; 5,141,813; 5,264,566; 4,959,463; 5,428,148; 5,554,744; 5,574,146; 5,602,244 each of which are incorporated herein by reference.

A non-limiting example of an enzymatically produced nucleic acid include nucleic acids produced by enzymes in amplification reactions such as PCR™ (see for example, U.S. Patents 4,683,202 and 4,682,195, each incorporated herein by reference), or the synthesis of an oligonucleotide described in U.S. Patent 5,645,897, incorporated herein by reference. A non-limiting example of a biologically produced nucleic acid includes a recombinant nucleic acid produced (*i.e.*, replicated) in a living cell, such as a recombinant DNA vector replicated in bacteria (see for example, Sambrook *et al.* 2001, incorporated herein by reference).

B. Purification of Nucleic Acids

A nucleic acid may be purified on polyacrylamide gels, cesium chloride centrifugation gradients, column chromatography or by any other means known to one of

ordinary skill in the art (see for example, Sambrook *et al.,* 2001, incorporated herein by reference). In certain aspects, the present invention concerns a nucleic acid that is an isolated nucleic acid. As used herein, the term "isolated nucleic acid" refers to a nucleic acid molecule (*e.g.*, an RNA or DNA molecule) that has been isolated free of, or is otherwise free of, bulk of cellular components or *in vitro* reaction components, and/or the bulk of the total genomic and transcribed nucleic acids of one or more cells. Methods for isolating nucleic acids (*e.g.*, equilibrium density centrifugation, electrophoretic separation, column chromatography) are well known to those of skill in the art.

V. Expression of Nucleic Acids

In accordance with the present invention, it will be desirable to produce p53 proteins in a cell. Expression typically requires that appropriate signals be provided in the vectors or expression cassettes, and which include various regulatory elements, such as enhancers/promoters from viral and/or mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells may also be included. Drug selection markers may be incorporated for establishing permanent, stable cell clones.

Viral vectors are selected eukaryotic expression systems. Included are adenoviruses, adeno-associated viruses, retroviruses, herpesviruses, lentivirus and poxviruses including vaccinia viruses and papilloma viruses including SV40. Viral vectors may be replication-defective, conditionally-defective or replication-competent. Also contemplated are non-viral delivery systems, including lipid-based vehicles.

A. Vectors and Expression Constructs

The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated and/or expressed. A nucleic acid sequence can be "exogenous" or "heterologous" which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and

artificial chromosomes (*e.g.,* YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (see, for example, Sambrook *et al.*, 2001 and Ausubel *et al.,* 1996, both incorporated herein by reference).

The term "expression vector" refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operable linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well, as described below.

In order to express p53, it is necessary to provide an expression vector. The appropriate nucleic acid can be inserted into an expression vector by standard subcloning techniques. The manipulation of these vectors is well known in the art. Examples of fusion protein expression systems are the glutathione S-transferase system (Pharmacia, Piscataway, NJ), the maltose binding protein system (NEB, Beverley, MA), the FLAG system (IBI, New Haven, CT), and the 6xHis system (Qiagen, Chatsworth, CA).

In yet another embodiment, the expression system used is one driven by the baculovirus polyhedron promoter. The gene encoding the protein can be manipulated by standard techniques in order to facilitate cloning into the baculovirus vector. A preferred baculovirus vector is the pBlueBac vector (Invitrogen, Sorrento, CA). The vector carrying the gene of interest is transfected into *Spodoptera frugiperda* (Sf9) cells by standard protocols, and the cells are cultured and processed to produce the recombinant protein. Mammalian cells exposed to baculoviruses become infected and may express the foreign gene only. This way one can transduce all cells and express the gene in dose dependent manner.

There also are a variety of eukaryotic vectors that provide a suitable vehicle in which recombinant polypeptide can be produced. HSV has been used in tissue culture to express a large number of exogenous genes as well as for high level expression of its endogenous genes. For example, the chicken ovalbumin gene has been expressed from

HSV using an α promoter. Herz and Roizman (1983). The *lacZ* gene also has been expressed under a variety of HSV promoters.

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of a gene and translation of a RNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid.

In preferred embodiments, the nucleic acid is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements

downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 1 and 2 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of a transgene. This list is not exhaustive of all the possible elements involved but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a

particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a transgene. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

PROMOTER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ ß
ß-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
ß-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein

PROMOTER
τ-Globin
ß-Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α_1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 2

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	Poly(rI)X Poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

One will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements (Bittner *et al.*, 1987).

In various embodiments of the invention, the expression construct may comprise a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986) and adeno-associated viruses. Retroviruses also are attractive gene transfer vehicles (Nicolas and Rubenstein, 1988; Temin, 1986) as are vaccinia virus (Ridgeway, 1988) and adeno-associated virus (Ridgeway, 1988). Such vectors may be used to (i) transform cell lines *in vitro* for the purpose of expressing proteins of interest or (ii) to transform cells *in vitro* or *in vivo* to provide therapeutic polypeptides in a gene therapy scenario.

B. Viral Vectors

Viral vectors are a kind of expression construct that utilizes viral sequences to introduce nucleic acid and possibly proteins into a cell. The ability of certain viruses to infect cells or enter cells *via* receptor-mediated endocytosis, and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign nucleic acids into cells (*e.g.*, mammalian cells). Vector components of the present invention may be a viral vector that encode one or

more candidate substance or other components such as, for example, an immunomodulator or adjuvant for the candidate substance. Non-limiting examples of virus vectors that may be used to deliver a nucleic acid of the present invention are described below.

1. Adenoviral Vectors

a. Virus Characteristics

Adenovirus is a non-enveloped double-stranded DNA virus. The virion consists of a DNA-protein core within a protein capsid. Virions bind to a specific cellular receptor, are endocytosed, and the genome is extruded from endosomes and transported to the nucleus. The genome is about 36 kB, encoding about 36 genes. In the nucleus, the "immediate early" E1A proteins are expressed initially, and these proteins induce expression of the "delayed early" proteins encoded by the E1B, E2, E3, and E4 transcription units. Virions assemble in the nucleus at about 1 day post infection (p.i.), and after 2-3 days the cell lyses and releases progeny virus. Cell lysis is mediated by the E3 11.6K protein, which has been renamed "adenovirus death protein" (ADP).

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

Adenovirus may be any of the 51 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the human adenovirus about which the most biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector. Recombinant adenovirus often is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Viruses used in gene therapy may be either replication-competent or replication-deficient. Generation and propagation of the adenovirus vectors which are replication-deficient depends on a helper cell line, the prototype being 293 cells, prepared by transforming human embryonic kidney cells with Ad5 DNA fragments; this cell line constitutively expresses E1 proteins (Graham *et al.*, 1977). However, helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.,* Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) have disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight,

following which the volume is increased to 100% and shaking commenced for another 72 h.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{13} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Animal studies have suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

b. Engineering

As stated above, Ad vectors are based on recombinant Ad's that are either replication-defective or replication-competent. Typical replication-defective Ad vectors lack the E1A and E1B genes (collectively known as E1) and contain in their place an expression cassette consisting of a promoter and pre-mRNA processing signals which drive expression of a foreign gene. These vectors are unable to replicate because they lack the E1A genes required to induce Ad gene expression and DNA replication. In addition, the E3 genes can be deleted because they are not essential for virus replication in cultured cells. It is recognized in the art that replication-defective Ad vectors have several characteristics that make them suboptimal for use in therapy. For example,

production of replication-defective vectors requires that they be grown on a complementing cell line that provides the E1A proteins *in trans*.

Several groups have also proposed using replication-competent Ad vectors for therapeutic use. Replication-competent vectors retain Ad genes essential for replication, and thus do not require complementing cell lines to replicate. Replication-competent Ad vectors lyse cells as a natural part of the life cycle of the vector. An advantage of replication-competent Ad vectors occurs when the vector is engineered to encode and express a foreign protein. Such vectors would be expected to greatly amplify synthesis of the encoded protein *in vivo* as the vector replicates. For use as anti-cancer agents, replication-competent viral vectors would theoretically be advantageous in that they would replicate and spread throughout the tumor, not just in the initially infected cells as is the case with replication-defective vectors.

Yet another approach is to create viruses that are conditionally-replication competent. Onyx Pharmaceuticals recently reported on adenovirus-based anti-cancer vectors which are replication-deficient in non-neoplastic cells, but which exhibit a replication phenotype in neoplastic cells lacking functional p53 and/or retinoblastoma (pRB) tumor suppressor proteins (U.S. Patent 5,677,178). This phenotype is reportedly accomplished by using recombinant adenoviruses containing a mutation in the E1B region that renders the encoded E1B-55K protein incapable of binding to p53 and/or a mutation(s) in the E1A region which make the encoded E1A protein (p289R or p243R) incapable of binding to pRB and/or p300 and/or p107. E1B-55K has at least two independent functions: it binds and inactivates the tumor suppressor protein p53, and it is required for efficient transport of Ad mRNA from the nucleus. Because these E1B and E1A viral proteins are involved in forcing cells into S-phase, which is required for replication of adenovirus DNA, and because the p53 and pRB proteins block cell cycle progression, the recombinant adenovirus vectors described by Onyx should replicate in cells defective in p53 and/or pRB, which is the case for many cancer cells, but not in cells with wild-type p53 and/or pRB.

Another replication-competent adenovirus vector has the gene for E1B-55K replaced with the herpes simplex virus thymidine kinase gene (Wilder *et al.*, 1999a). The group that constructed this vector reported that the combination of the vector plus

gancyclovir showed a therapeutic effect on a human colon cancer in a nude mouse model (Wilder *et al.*, 1999b). However, this vector lacks the gene for ADP, and accordingly, the vector will lyse cells and spread from cell-to-cell less efficiently than an equivalent vector that expresses ADP.

The present inventor has taken advantage of the differential expression of telomerase in dividing cells to create novel adenovirus vectors which overexpress an adenovirus death protein and which are replication-competent in and, preferably, replication-restricted to cells expressing telomerase. Specific embodiments include disrupting E1A's ability to bind p300 and/or members of the Rb family members. Others include Ad vectors lacking expression of at least one E3 protein selected from the group consisting of 6.7K, gp19K, RIDα (also known as 10.4K); RIDβ (also known as 14.5K) and 14.7K. Because wild-type E3 proteins inhibit immune-mediated inflammation and/or apoptosis of Ad-infected cells, a recombinant adenovirus lacking one or more of these E3 proteins may stimulate infiltration of inflammatory and immune cells into a tumor treated with the adenovirus and that this host immune response will aid in destruction of the tumor as well as tumors that have metastasized. A mutation in the E3 region would impair its wild-type function, making the viral-infected cell susceptible to attack by the host's immune system. These viruses are described in detail in U.S. Patent 6,627,190.

Other adenoviral vectors are described in U.S. Patents 5,670,488; 5,747,869; 5,932,210; 5,981,225; 6,069,134; 6,136,594; 6,143,290; 6,210,939; 6,296,845; 6,410,010; and 6,511,184; U.S. Publication No. 2002/0028785.

2. AAV Vectors

The nucleic acid may be introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems (Kelleher and Vos, 1994; Cotten *et al.*, 1992; Curiel, 1994). Adeno-associated virus (AAV) is an attractive vector system for use in the methods of the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells, for example, in tissue culture (Muzyczka, 1992) or *in vivo*. AAV has a broad host range for infectivity (Tratschin *et al.*, 1984; Laughlin *et al.*, 1986; Lebkowski *et al.*, 1988; McLaughlin *et*

al., 1988). Details concerning the generation and use of rAAV vectors are described in U.S. Patents 5,139,941 and 4,797,368, each incorporated herein by reference.

3. Retroviral Vectors

Retroviruses have promise as therapeutic vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines (Miller, 1992).

In order to construct a retroviral vector, a nucleic acid is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into a special cell line (*e.g.*, by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes *gag*, *pol*, and *env*, contain other genes with regulatory or structural function. Lentiviral vectors are well known in the art (see, for example, Naldini *et al.*, 1996; Zufferey *et al.*, 1997; Blomer *et al.*, 1997; U.S. Patents 6,013,516 and 5,994,136).

Recombinant lentiviral vectors are capable of infecting non-dividing cells and can be used for both *in vivo* and *ex vivo* gene transfer and expression of nucleic acid sequences. For example, recombinant lentivirus capable of infecting a non-dividing cell wherein a suitable host cell is transfected with two or more vectors carrying the packaging functions, namely gag, pol and env, as well as rev and tat is described in U.S. Patent 5,994,136, incorporated herein by reference. One may target the recombinant

virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including a regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific.

4. Other Viral Vectors

Other viral vectors may be employed as vaccine constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988), sindbis virus, cytomegalovirus and herpes simplex virus may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

5. Delivery Using Modified Viruses

A nucleic acid to be delivered may be housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

6. Non-Viral Delivery

Lipid-based non-viral formulations provide an alternative to adenoviral gene therapies. Although many cell culture studies have documented lipid-based non-viral gene transfer, systemic gene delivery via lipid-based formulations has been limited. A major limitation of non-viral lipid-based gene delivery is the toxicity of the cationic lipids that comprise the non-viral delivery vehicle. The *in vivo* toxicity of liposomes partially explains the discrepancy between *in vitro* and *in vivo* gene transfer results. Another factor contributing to this contradictory data is the difference in liposome stability in the presence and absence of serum proteins. The interaction between liposomes and serum proteins has a dramatic impact on the stability characteristics of liposomes (Yang and Huang, 1997). Cationic liposomes attract and bind negatively charged serum proteins. Liposomes coated by serum proteins are either dissolved or taken up by macrophages leading to their removal from circulation. Current *in vivo* liposomal delivery methods use aerosolization, subcutaneous, intradermal, intratumoral, or intracranial injection to avoid the toxicity and stability problems associated with cationic lipids in the circulation. The interaction of liposomes and plasma proteins islargely responsible for the disparity between the efficiency of *in vitro* (Felgner *et al.*, 1987) and *in vivo* gene transfer (Zhu *et al.*, 1993; Philip *et al.*, 1993; Solodin *et al.*, 1995; Liu *et al.*, 1995; Thierry *et al.*, 1995; Tsukamoto *et al.*, 1995; Aksentijevich *et al.*, 1996).

Recent advances in liposome formulations have improved the efficiency of gene transfer *in vivo* (Templeton *et al.* 1997; WO 98/07408, incorporated herein by reference). A novel liposomal formulation composed of an equimolar ratio of 1,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP) and cholesterol significantly enhances systemic *in vivo* gene transfer, approximately 150 fold. The DOTAP:cholesterol lipid formulation is said to form a unique structure termed a "sandwich liposome." This formulation is reported to "sandwich" DNA between an invaginated bilayer or "vase" structure. Beneficial characteristics of these liposomes include a positive to negative charge or ρ, colloidal stabilization by cholesterol, two-dimensional DNA packing and increased serum stability.

The production of lipid formulations often is accomplished by sonication or serial extrusion of liposomal mixtures after (I) reverse phase evaporation (II) dehydration-rehydration (III) detergent dialysis and (IV) thin film hydration. Once manufactured, lipid structures can be used to encapsulate compounds that are toxic (chemotherapeutics) or labile (nucleic acids) when in circulation. Liposomal encapsulation has resulted in a lower toxicity and a longer serum half-life for such compounds (Gabizon *et al.,* 1990). Numerous disease treatments are using lipid based gene transfer strategies to enhance conventional or establish novel therapies, in particular therapies for treating hyperproliferative diseases.

Liposomes are vesicular structures characterized by a lipid bilayer and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when lipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of structures that entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Lipophilic molecules or molecules with lipophilic regions may also dissolve in or associate with the lipid bilayer.

The liposomes are capable of carrying biologically active nucleic acids, such that the nucleic acids are completely sequestered. The liposome may contain one or more nucleic acids and is administered to a mammalian host to efficiently deliver its contents to a target cell. The liposomes may comprise DOTAP and cholesterol or a cholesterol derivative. In certain embodiments, the ratio of DOTAP to cholesterol, cholesterol derivative or cholesterol mixture is about 10:1 to about 1:10, about 9:1 to about 1:9, about 8:1 to about 1:8, about 7:1 to about 1:7, about 6:1 to about 1:6, about 5:1 to about 1:5, about 4:1 to about 1:4, about 3:1 to 1:3, more preferably 2:1 to 1:2, and most preferably 1:1. In further preferred embodiments, the DOTAP and/or cholesterol concentrations are about 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM, 25 mM, or 30 mM. The DOTAP and/or Cholesterol concentration can be between about 1 mM to about 20mM, 1 mM to about 18 mM, 1 mM to about 16 mM, about 1 mM to about 14 mM, about 1 mM to about 12 mM, about 1 mM to about 10 mM, 1 to 8 mM, more preferably 2 to 7 mM, still more preferably 3 to 6 mM and most

preferably 4 to 5 mM. Cholesterol derivatives may be readily substituted for the cholesterol or mixed with the cholesterol in the present invention. Many cholesterol derivatives are known to the skilled artisan. Examples include but are not limited to cholesterol acetate and cholesterol oleate. A cholesterol mixture refers to a composition that contains at least one cholesterol or cholesterol derivative.

The formulation may also be extruded using a membrane or filter, and this may be performed multiple times. Such techniques are well-known to those of skill in the art, for example in Martin (1990). Extrusion may be performed to homogenize the formulation or limit its size. A contemplated method for preparing liposomes in certain embodiments is heating, sonicating, and sequential extrusion of the lipids through filters of decreasing pore size, thereby resulting in the formation of small, stable liposome structures. This preparation produces liposomal complexesor liposomes only of appropriate and uniform size, which are structurally stable and produce maximal activity.

For example, it is contemplated in certain embodiments of the present invention that DOTAP:Cholesterol liposomes are prepared by the methods of Templeton *et al.* (1997; incorporated herein by reference). Thus, in one embodiment, DOTAP (cationic lipid) is mixed with cholesterol (neutral lipid) at equimolar concentrations. This mixture of powdered lipids is then dissolved with chloroform, the solution dried to a thin film and the film hydrated in water containing 5% dextrose (w/v) to give a final concentration of 20 mM DOTAP and 20 mM cholesterol. The hydrated lipid film is rotated in a 50°C water bath for 45 minutes, then at 35°C for an additional 10 minutes and left standing at room temperature overnight. The following day the mixture is sonicated for 5 minutes at 50°C. The sonicated mixture is transferred to a tube and heated for 10 minutes at 50°C. This mixture is sequentially extruded through syringe filters of decreasing pore size (1 μm, 0.45 μm, 0.2 μm, 0.1 μm).

It also is contemplated that other liposome formulations and methods of preparation may be combined to impart desired DOTAP:Cholesterol liposome characteristics. Alternate methods of preparing lipid-based formulations for nucleic acid delivery are described by Saravolac *et al.* (W0 99/18933). Detailed are methods in which lipids compositions are formulated specifically to encapsulate nucleic acids. In another liposome formulation, an amphipathic vehicle called a solvent dilution microcarrier

(SDMC) enables integration of particular molecules into the bi-layer of the lipid vehicle (U.S. Patent 5,879,703). The SDMCs can be used to deliver lipopolysaccharides, polypeptides, nucleic acids and the like. Of course, any other methods of liposome preparation can be used by the skilled artisan to obtain a desired liposome formulation in the present invention.

C. Vector Delivery and Cell Transformation

Suitable methods for nucleic acid delivery for transformation of an organelle, a cell, a tissue or an organism for use with the current invention are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by *ex vivo* transfection (Wilson *et al.*, 1989; Nabel *et al.*, 1989), by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harlan and Weintraub, 1985; U.S. Patent 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent 5,384,253, incorporated herein by reference; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988); by microprojectile bombardment (WO 94/09699 and WO 95/06128; U.S. Patents 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppler *et al.*, 1990; U.S. Patents 5,302,523 and 5,464,765, each incorporated herein by reference); and any combination of such methods.

D. Expression Systems

Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patents. 5,871,986, 4,879,236, both herein incorporated by reference, and which can be bought, for example, under the name MAXBAC® 2.0 from INVITROGEN® and BACPACK™ BACULOVIRUS EXPRESSION SYSTEM FROM CLONTECH®.

Other examples of expression systems include STRATAGENE®'s COMPLETE CONTROL™ Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN® also provides a yeast expression system called the *Pichia methanolica* Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

It is contemplated that p53 may be "overexpressed," *i.e.*, expressed in increased levels relative to its natural expression in cells. Such overexpression may be assessed by a variety of methods, including radio-labeling and/or protein purification. However, simple and direct methods are preferred, for example, those involving SDS/PAGE and protein staining or western blotting, followed by quantitative analyses, such as densitometric scanning of the resultant gel or blot. A specific increase in the level of the recombinant protein, polypeptide or peptide in comparison to the level in natural cells is indicative of overexpression, as is a relative abundance of the specific protein,

polypeptides or peptides in relation to the other proteins produced by the host cell, *e.g.*, visible on a gel.

In some embodiments, the expressed proteinaceous sequence forms an inclusion body in the host cell, the host cells are lysed, for example, by disruption in a cell homogenizer, washed and/or centrifuged to separate the dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby the dense inclusion bodies are selectively enriched by incorporation of sugars, such as sucrose, into the buffer and centrifugation at a selective speed. Inclusion bodies may be solubilized in solutions containing high concentrations of urea (*e.g.*, 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents, such as β-mercaptoethanol or DTT (dithiothreitol), and refolded into a more desirable conformation, as would be known to one of ordinary skill in the art.

The nucleotide and protein sequences for p53 have been previously disclosed, and may be found at computerized databases known to those of ordinary skill in the art. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (www.ncbi.nlm.nih.gov/). The coding regions for these known genes may be amplified and/or expressed using the techniques disclosed herein or by any technique that would be known to those of ordinary skill in the art. Additionally, peptide sequences may be synthesized by methods known to those of ordinary skill in the art, such as peptide synthesis using automated peptide synthesis machines, such as those available from Applied Biosystems (Foster City, CA).

E. Multigene Constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picanovirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be

transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

VI. Therapeutic Intervention

In accordance with the present invention, applicants provide methods for treating recurrent cancer, particularly cancer that has recurred following surgery, radio- and/or chemotherapy. More particularly, the invention relates to treating recurrent cancers with a subsequent radio and/or chemotherapy regimen or agent by administering to a patient and expression construct encoding p53. U.S. Patent 5,747,469, U.S. Application No. 2002/0006914, and U.S. Application No. 2002/0077313, each of which disclose p53 therapies in combination with radio- and chemotherapies, are hereby incorporated by reference. In a particular embodiment, the radio and/or chemotherapy incorporates a DNA-damaging regimen or agent.

The radio- or chemotherapy that is provided subsequent to p53 gene therapy may occur relatively quickly, although long enough after the p53 gene therapy to permit p53 expression. Thus, it is contemplated that earlier time points for subsequent therapy include as early as about 24 hours post-p53 treatment. However, beneficial effects have been seen at much long times following p53 treatment, for example in the 3- to 6-month time frame. Thus, the present invention contemplates times periods between p53 and subsequent radio- or chemotherapy of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days, three, four, five, six, seven or eight weeks, one two, three four, five, or six months, and up to one year.

The present invention may be utilized in a variety of solid cancers, such as brain cancer, head & neck cancer, esophageal cancer, tracheal cancer, lung cancer, liver cancer stomach cancer, colon cancer, pancreatic cancer, breast cancer, cervical cancer, uterine cancer, bladder cancer, prostate cancer, testicular cancer, skin cancer or rectal cancer. It also may be used against lymphomas or leukemias.

Local, region or systemic delivery of p53 expression constructs and/or chemotherapeutic drugs and/or radiation to patients is contemplated. It is proposed that

this approach will provide clinical benefit, defined broadly as any of the following: reducing primary tumor size, reducing occurrence or size of metastasis, reducing or stopping tumor growth, inhibiting tumor cell division, killing a tumor cell, inducing apoptosis in a tumor cell, reducing or eliminating tumor recurrence.

Patients with unresectable tumors may be treated according to the present invention. As a consequence, the tumor may reduce in size, or the tumor vasculature may change such that the tumor becomes resectable. If so, standard surgical resection may be permitted.

A. Recurrent Cancer

An cancer recurrence may be defined a the reappearance or rediagnosis of a patent as having any cancer following one or more of surgery, radiotherapy or chemotherapy. The patient need not have been reported as disease free, but merely that the patient has exhibited renewed cancer growth following some degree of clinical response by the first therapy. The clinical response may be, but is not limited to, stable disease, tumor regression, tumor necrosis, or absence of demonstrable cancer.

B. p53 Gene Therapy

Human p53 gene therapy has been described in the literature since the mid-1990's. Roth *et al.* (1996) reported on retroviral-based therapy, Clayman *et al.* (1998) described adenoviral delivery. U.S. Patents 6,017,524; 6,143,290; 6,410,010; and 6,511,847, and U.S. Patent Application No. 2002/0077313 each describe methods of treating patients with p53, and are hereby incorporated by reference.

One particular mode of administration that can be used in conjunction with surgery is treatment of an operative tumor bed. Thus, in either the primary gene therapy treatment, or in a subsequent treatment, one may perfuse the resected tumor bed with the vector during surgery, and following surgery, optionally by inserting a catheter into the surgery site.

C. Chemotherapy

A wide variety of chemotherapeutic agents may be used in accordance with the present invention. The term "chemotherapy" refers to the use of drugs to treat cancer. A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis. Most chemotherapeutic agents fall into the following categories: alkylating agents, antimetabolites, antitumor antibiotics, mitotic inhibitors, and nitrosoureas.

1. Alkylating agents

Alkylating agents are drugs that directly interact with genomic DNA to prevent the cancer cell from proliferating. This category of chemotherapeutic drugs represents agents that affect all phases of the cell cycle, that is, they are not phase-specific. Alkylating agents can be implemented to treat chronic leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, and particular cancers of the breast, lung, and ovary. They include: busulfan, chlorambucil, cisplatin, cyclophosphamide (cytoxan), dacarbazine, ifosfamide, mechlorethamine (mustargen), and melphalan. Troglitazaone can be used to treat cancer in combination with any one or more of these alkylating agents, some of which are discussed below.

a. Busulfan

Busulfan (also known as myleran) is a bifunctional alkylating agent. Busulfan is known chemically as 1,4-butanediol dimethanesulfonate.

Busulfan is not a structural analog of the nitrogen mustards. Busulfan is available in tablet form for oral administration. Each scored tablet contains 2 mg busulfan and the inactive ingredients magnesium stearate and sodium chloride.

Busulfan is indicated for the palliative treatment of chronic myelogenous (myeloid, myelocytic, granulocytic) leukemia. Although not curative, busulfan reduces

the total granulocyte mass, relieves symptoms of the disease, and improves the clinical state of the patient. Approximately 90% of adults with previously untreated chronic myelogenous leukemia will obtain hematologic remission with regression or stabilization of organomegaly following the use of busulfan. It has been shown to be superior to splenic irradiation with respect to survival times and maintenance of hemoglobin levels, and to be equivalent to irradiation at controlling splenomegaly.

b. Chlorambucil

Chlorambucil (also known as leukeran) is a bifunctional alkylating agent of the nitrogen mustard type that has been found active against selected human neoplastic diseases. Chlorambucil is known chemically as 4-[bis(2-chlorethyl)amino] benzenebutanoic acid.

Chlorambucil is available in tablet form for oral administration. It is rapidly and completely absorbed from the gastrointestinal tract. After single oral doses of 0.6-1.2 mg/kg, peak plasma chlorambucil levels are reached within one hour and the terminal half-life of the parent drug is estimated at 1.5 hours. 0.1 to 0.2mg/kg/day or 3 to 6mg/m^2/day or alternatively 0.4mg/kg may be used for antineoplastic treatment. Treatment regimes are well know to those of skill in the art and can be found in the "Physicians Desk Reference" and in "Remington's Pharmaceutical Sciences" referenced herein.

Chlorambucil is indicated in the treatment of chronic lymphatic (lymphocytic) leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma and Hodgkin's disease. It is not curative in any of these disorders but may produce clinically useful palliation. Thus, it can be used in combination with troglitazone in the treatment of cancer.

c. Cisplatin

Cisplatin has been widely used to treat cancers such as metastatic testicular or ovarian carcinoma, advanced bladder cancer, head or neck cancer, cervical cancer, lung cancer or other tumors. Cisplatin can be used alone or in combination with other agents, with efficacious doses used in clinical applications of 15-20 mg/m^2 for 5 days every three

weeks for a total of three courses. Exemplary doses may be 0.50 mg/m^2, 1.0mg/m^2, 1.50 mg/m^2, 1.75 mg/m^2, 2.0 mg/m^2, 3.0 mg/m^2 , 4.0 mg/m^2, 5.0 mg/m^2 , 10mg//m^2. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

Cisplatin is not absorbed orally and must therefore be delivered *via* injection intravenously, subcutaneously, intratumorally or intraperitoneally.

d. Cyclophosphamide

Cyclophosphamide is 2*H*-1,3,2-Oxazaphosphorin-2-amine, *N*,*N*-bis(2-chloroethyl)tetrahydro-, 2-oxide, monohydrate; termed Cytoxan available from Mead Johnson; and Neosar available from Adria. Cyclophosphamide is prepared by condensing 3-amino-1-propanol with *N*,*N*-bis(2-chlorethyl) phosphoramidic dichloride [$(ClCH_2CH_2)_2N$--$POCl_2$] in dioxane solution under the catalytic influence of triethylamine. The condensation is double, involving both the hydroxyl and the amino groups, thus effecting the cyclization.

Unlike other ß-chloroethylamino alkylators, it does not cyclize readily to the active ethyleneimonium form until activated by hepatic enzymes. Thus, the substance is stable in the gastrointestinal tract, tolerated well and effective by the oral and parental routes and does not cause local vesication, necrosis, phlebitis or even pain.

Suitable doses for adults include, orally, 1 to 5 mg/kg/day (usually in combination), depending upon gastrointestinal tolerance; or 1 to 2 mg/kg/day; intravenously, initially 40 to 50 mg/kg in divided doses over a period of 2 to 5 days or 10 to 15 mg/kg every 7 to 10 days or 3 to 5 mg/kg twice a week or 1.5 to 3 mg/kg/day . A dose 250mg/kg/day may be administered as an antineoplastic. Because of gastrointestinal adverse effects, the intravenous route is preferred for loading. During maintenance, a leukocyte count of 3000 to 4000/mm^3 usually is desired. The drug also sometimes is administered intramuscularly, by infiltration or into body cavities. It is available in dosage forms for injection of 100, 200 and 500 mg, and tablets of 25 and 50 mg the skilled artisan is referred to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61, incorporate herein as a reference, for details on doses for administration.

e. Melphalan

Melphalan, also known as alkeran, L-phenylalanine mustard, phenylalanine mustard, L-PAM, or L-sarcolysin, is a phenylalanine derivative of nitrogen mustard. Melphalan is a bifunctional alkylating agent which is active against selective human neoplastic diseases. It is known chemically as 4-[bis(2-chloroethyl)amino]-L-phenylalanine.

Melphalan is the active L-isomer of the compound and was first synthesized in 1953 by Bergel and Stock; the D-isomer, known as medphalan, is less active against certain animal tumors, and the dose needed to produce effects on chromosomes is larger than that required with the L-isomer. The racemic (DL-) form is known as merphalan or sarcolysin. Melphalan is insoluble in water and has a pKa_1 of ~2.1. Melphalan is available in tablet form for oral administration and has been used to treat multiple myeloma.

Available evidence suggests that about one third to one half of the patients with multiple myeloma show a favorable response to oral administration of the drug.

Melphalan has been used in the treatment of epithelial ovarian carcinoma. One commonly employed regimen for the treatment of ovarian carcinoma has been to administer melphalan at a dose of 0.2 mg/kg daily for five days as a single course. Courses are repeated every four to five weeks depending upon hematologic tolerance (Smith and Rutledge, 1975; Young *et al.*, 1978). Alternatively the dose of melphalan used could be as low as 0.05mg/kg/day or as high as 3mg/kg/day or any dose in between these doses or above these doses. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject

2. Antimetabolites

Antimetabolites disrupt DNA and RNA synthesis. Unlike alkylating agents, they specifically influence the cell cycle during S phase. They have used to combat chronic leukemias in addition to tumors of breast, ovary and the gastrointestinal tract.

Antimetabolites include 5-fluorouracil (5-FU), cytarabine (Ara-C), fludarabine, gemcitabine, and methotrexate.

5-Fluorouracil (5-FU) has the chemical name of 5-fluoro-2,4(1H,3H)-pyrimidinedione. Its mechanism of action is thought to be by blocking the methylation reaction of deoxyuridylic acid to thymidylic acid. Thus, 5-FU interferes with the syntheisis of deoxyribonucleic acid (DNA) and to a lesser extent inhibits the formation of ribonucleic acid (RNA). Since DNA and RNA are essential for cell division and proliferation, it is thought that the effect of 5-FU is to create a thymidine deficiency leading to cell death. Thus, the effect of 5-FU is found in cells that rapidly divide, a characteristic of metastatic cancers.

3. Antitumor Antibiotics

Antitumor antibiotics have both antimicrobial and cytotoxic activity. These drugs also interfere with DNA by chemically inhibiting enzymes and mitosis or altering cellular membranes. These agents are not phase specific so they work in all phases of the cell cycle. Thus, they are widely used for a variety of cancers. Examples of antitumor antibiotics include bleomycin, dactinomycin, daunorubicin, doxorubicin (Adriamycin), and idarubicin, some of which are discussed in more detail below. Widely used in clinical setting for the treatment of neoplasms these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m^2 at 21 day intervals for adriamycin, to 35-100 mg/m^2 for etoposide intravenously or orally.

a. Doxorubicin

Doxorubicin hydrochloride, 5,12-Naphthacenedione, (8s-*cis*)-10-[(3-amino-2,3,6-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-hydrochloride (hydroxydaunorubicin hydrochloride, Adriamycin) is used in a wide antineoplastic spectrum. It binds to DNA and inhibits nucleic acid synthesis, inhibits mitosis and promotes chromosomal aberrations.

Administered alone, it is the drug of first choice for the treatment of thyroid adenoma and primary hepatocellular carcinoma. It is a component of 31 first-choice combinations for the treatment of ovarian, endometrial and breast tumors, bronchogenic

oat-cell carcinoma, non-small cell lung carcinoma, gastric adenocarcinoma, retinoblastoma, neuroblastoma, mycosis fungoides, pancreatic carcinoma, prostatic carcinoma, bladder carcinoma, myeloma, diffuse histiocytic lymphoma, Wilms' tumor, Hodgkin's disease, adrenal tumors, osteogenic sarcoma soft tissue sarcoma, Ewing's sarcoma, rhabdomyosarcoma and acute lymphocytic leukemia. It is an alternative drug for the treatment of islet cell, cervical, testicular and adrenocortical cancers. It is also an immunosuppressant.

Doxorubicin is absorbed poorly and must be administered intravenously. The pharmacokinetics are multicompartmental. Distribution phases have half-lives of 12 minutes and 3.3 hr. The elimination half-life is about 30 hr. Forty to 50% is secreted into the bile. Most of the remainder is metabolized in the liver, partly to an active metabolite (doxorubicinol), but a few percent is excreted into the urine. In the presence of liver impairment, the dose should be reduced.

Appropriate doses are, intravenous, adult, 60 to 75 mg/m^2 at 21-day intervals or 25 to 30 mg/m^2 on each of 2 or 3 successive days repeated at 3- or 4-wk intervals or 20 mg/m^2 once a week. The lowest dose should be used in elderly patients, when there is prior bone-marrow depression caused by prior chemotherapy or neoplastic marrow invasion, or when the drug is combined with other myelopoietic suppressant drugs. The dose should be reduced by 50% if the serum bilirubin lies between 1.2 and 3 mg/dL and by 75% if above 3 mg/dL. The lifetime total dose should not exceed 550 mg/m^2 in patients with normal heart function and 400 mg/m^2 in persons having received mediastinal irradiation. Alternatively, 30 mg/m^2 on each of 3 consecutive days, repeated every 4 wk. Exemplary doses may be 10 mg/m^2, 20 mg/m^2, 30 mg/m^2, 50 mg/m^2, 100 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 225 mg/m^2, 250 mg/m^2, 275 mg/m^2, 300 mg/m^2, 350 mg/m^2, 400 mg/m^2, 425 mg/m^2, 450 mg/m^2, 475 mg/m^2, 500 mg/m^2. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

In the present invention the inventors have employed troglitazone as an exemplary chemotherapeutic agent to synergistically enhance the antineoplastic effects of the doxorubicin in the treatment of cancers. Those of skill in the art will be able to use the

invention as exemplified potentiate the effects of doxorubicin in a range of different pre-cancer and cancers.

b. Daunorubicin

Daunorubicin hydrochloride, 5,12-Naphthacenedione, (8S-*cis*)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-a-L-lyxo-hexanopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-10-methoxy-, hydrochloride; also termed cerubidine and available from Wyeth. Daunorubicin intercalates into DNA, blocks DAN-directed RNA polymerase and inhibits DNA synthesis. It can prevent cell division in doses that do not interfere with nucleic acid synthesis.

In combination with other drugs it is included in the first-choice chemotherapy of acute myelocytic leukemia in adults (for induction of remission), acute lymphocytic leukemia and the acute phase of chronic myelocytic leukemia. Oral absorption is poor, and it must be given intravenously. The half-life of distribution is 45 minutes and of elimination, about 19 hr. The half-life of its active metabolite, daunorubicinol, is about 27 hr. Daunorubicin is metabolized mostly in the liver and also secreted into the bile (ca 40%). Dosage must be reduced in liver or renal insufficiencies.

Suitable doses are (base equivalent), intravenous adult, younger than 60 yr. 45 mg/m^2/day (30 mg/m^2 for patients older than 60 yr.) for 1, 2 or 3 days every 3 or 4 wk or 0.8 mg/kg/day for 3 to 6 days every 3 or 4 wk; no more than 550 mg/m^2 should be given in a lifetime, except only 450 mg/m^2 if there has been chest irradiation; children, 25 mg/m^2 once a week unless the age is less than 2 yr. or the body surface less than 0.5 m, in which case the weight-based adult schedule is used. It is available in injectable dosage forms (base equivalent) 20 mg (as the base equivalent to 21.4 mg of the hydrochloride). Exemplary doses may be 10 mg/m^2, 20 mg/m^2, 30 mg/m^2, 50 mg/m^2, 100 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 225 mg/m^2, 250 mg/m^2, 275 mg/m^2, 300 mg/m^2, 350 mg/m^2, 400 mg/m^2, 425 mg/m^2, 450 mg/m^2, 475 mg/m^2, 500 mg/m^2. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

c. Mitomycin

Mitomycin (also known as mutamycin and/or mitomycin-C) is an antibiotic isolated from the broth of *Streptomyces caespitosus* which has been shown to have antitumor activity. The compound is heat stable, has a high melting point, and is freely soluble in organic solvents.

Mitomycin selectively inhibits the synthesis of deoxyribonucleic acid (DNA). The guanine and cytosine content correlates with the degree of mitomycin-induced cross-linking. At high concentrations of the drug, cellular RNA and protein synthesis are also suppressed.

In humans, mitomycin is rapidly cleared from the serum after intravenous administration. Time required to reduce the serum concentration by 50% after a 30 mg. bolus injection is 17 minutes. After injection of 30 mg., 20 mg., or 10 mg. I.V., the maximal serum concentrations were 2.4 mg./mL, 1.7 mg./mL, and 0.52 mg./mL, respectively. Clearance is effected primarily by metabolism in the liver, but metabolism occurs in other tissues as well. The rate of clearance is inversely proportional to the maximal serum concentration because, it is thought, of saturation of the degradative pathways. Approximately 10% of a dose of mitomycin is excreted unchanged in the urine. Since metabolic pathways are saturated at relatively low doses, the percent of a dose excreted in urine increases with increasing dose. In children, excretion of intravenously administered mitomycin is similar.

d. Actinomycin D

Actinomycin D (Dactinomycin) [50-76-0]; $C_{62}H_{86}N_{12}O_{16}$ (1255.43) is an antineoplastic drug that inhibits DNA-dependent RNA polymerase. It is a component of first-choice combinations for treatment of choriocarcinoma, embryonal rhabdomyosarcoma, testicular tumor and Wilms' tumor. Tumors that fail to respond to systemic treatment sometimes respond to local perfusion. Dactinomycin potentiates radiotherapy. It is a secondary (efferent) immunosuppressive.

Actinomycin D is used in combination with primary surgery, radiotherapy, and other drugs, particularly vincristine and cyclophosphamide. Antineoplastic activity has also been noted in Ewing's tumor, Kaposi's sarcoma, and soft-tissue sarcomas.

Dactinomycin can be effective in women with advanced cases of choriocarcinoma. It also produces consistent responses in combination with chlorambucil and methotrexate in patients with metastatic testicular carcinomas. A response may sometimes be observed in patients with Hodgkin's disease and non-Hodgkin's lymphomas. Dactinomycin has also been used to inhibit immunological responses, particularly the rejection of renal transplants.

Half of the dose is excreted intact into the bile and 10% into the urine; the half-life is about 36 hr. The drug does not pass the blood-brain barrier. Actinomycin D is supplied as a lyophilized powder (0/5 mg in each vial). The usual daily dose is 10 to 15 mg/kg; this is given intravenously for 5 days; if no manifestations of toxicity are encountered, additional courses may be given at intervals of 3 to 4 weeks. Daily injections of 100 to 400 mg have been given to children for 10 to 14 days; in other regimens, 3 to 6 mg/kg, for a total of 125 mg/kg, and weekly maintenance doses of 7.5 mg/kg have been used. Although it is safer to administer the drug into the tubing of an intravenous infusion, direct intravenous injections have been given, with the precaution of discarding the needle used to withdraw the drug from the vial in order to avoid subcutaneous reaction. Exemplary doses may be 100 mg/m^2, 150 mg/m^2, 175 mg/m^2, 200 mg/m^2, 225 mg/m^2, 250 mg/m^2, 275 mg/m^2, 300 mg/m^2, 350 mg/m^2, 400 mg/m^2, 425 mg/m^2, 450 mg/m^2, 475 mg/m^2, 500 mg/m^2. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

e. Bleomycin

Bleomycin is a mixture of cytotoxic glycopeptide antibiotics isolated from a strain of *Streptomyces verticillus*. Although the exact mechanism of action of bleomycin is unknown, available evidence would seem to indicate that the main mode of action is the inhibition of DNA synthesis with some evidence of lesser inhibition of RNA and protein synthesis.

In mice, high concentrations of bleomycin are found in the skin, lungs, kidneys, peritoneum, and lymphatics. Tumor cells of the skin and lungs have been found to have high concentrations of bleomycin in contrast to the low concentrations found in

hematopoietic tissue. The low concentrations of bleomycin found in bone marrow may be related to high levels of bleomycin degradative enzymes found in that tissue.

In patients with a creatinine clearance of >35 mL per minute, the serum or plasma terminal elimination half-life of bleomycin is approximately 115 minutes. In patients with a creatinine clearance of <35 mL per minute, the plasma or serum terminal elimination half-life increases exponentially as the creatinine clearance decreases. In humans, 60% to 70% of an administered dose is recovered in the urine as active bleomycin. Bleomycin may be given by the intramuscular, intravenous, or subcutaneous routes. It is freely soluble in water.

Bleomycin should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents in squamous cell carcinoma such as head and neck (including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, larynx), skin, penis, cervix, and vulva. It has also been used in the treatment of lymphomas and testicular carcinoma.

Because of the possibility of an anaphylactoid reaction, lymphoma patients should be treated with two units or less for the first two doses. If no acute reaction occurs, then the regular dosage schedule may be followed.

Improvement of Hodgkin's Disease and testicular tumors is prompt and noted within 2 weeks. If no improvement is seen by this time, improvement is unlikely. Squamous cell cancers respond more slowly, sometimes requiring as long as 3 weeks before any improvement is noted.

4. Mitotic Inhibitors

Mitotic inhibitors include plant alkaloids and other natural agents that can inhibit either protein synthesis required for cell division or mitosis. They operate during a specific phase during the cell cycle. Mitotic inhibitors comprise docetaxel, etoposide (VP16), paclitaxel, taxol, taxotere, vinblastine, vincristine, and vinorelbine.

a. Etoposide (VP16)

VP16 is also known as etoposide and is used primarily for treatment of testicular tumors, in combination with bleomycin and cisplatin, and in combination with cisplatin for small-cell carcinoma of the lung. It is also active against non-Hodgkin's lymphomas, acute nonlymphocytic leukemia, carcinoma of the breast, and Kaposi's sarcoma associated with acquired immunodeficiency syndrome (AIDS).

VP16 is available as a solution (20 mg/ml) for intravenous administration and as 50-mg, liquid-filled capsules for oral use. For small-cell carcinoma of the lung, the intravenous dose (in combination therapy) is can be as much as 100 mg/m^2 or as little as 2 mg/ m^2, routinely 35 mg/m^2, daily for 4 days, to 50 mg/m^2, daily for 5 days have also been used. When given orally, the dose should be doubled. Hence the doses for small cell lung carcinoma may be as high as 200-250mg/m^2. The intravenous dose for testicular cancer (in combination therapy) is 50 to 100 mg/m^2 daily for 5 days, or 100 mg/m^2 on alternate days, for three doses. Cycles of therapy are usually repeated every 3 to 4 weeks. The drug should be administered slowly during a 30- to 60-minute infusion in order to avoid hypotension and bronchospasm, which are probably due to the solvents used in the formulation.

b. Taxol

Taxol is an experimental antimitotic agent, isolated from the bark of the ash tree, *Taxus brevifolia*. It binds to tubulin (at a site distinct from that used by the vinca alkaloids) and promotes the assembly of microtubules. Taxol is currently being evaluated clinically; it has activity against malignant melanoma and carcinoma of the ovary. Maximal doses are 30 mg/m^2 per day for 5 days or 210 to 250 mg/m^2 given once every 3 weeks. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

c. Vinblastine

Vinblastine is another example of a plant aklyloid that can be used in combination with troglitazone for the treatment of cancer and precancer. When cells are incubated with vinblastine, dissolution of the microtubules occurs.

Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each drug in plasma is approximately 0.4 mM. Vinblastine and vincristine bind to plasma proteins. They are extensively concentrated in platelets and to a lesser extent in leukocytes and erythrocytes.

After intravenous injection, vinblastine has a multiphasic pattern of clearance from the plasma; after distribution, drug disappears from plasma with half-lives of approximately 1 and 20 hours. Vinblastine is metabolized in the liver to biologically activate derivative desacetylvinblastine. Approximately 15% of an administered dose is detected intact in the urine, and about 10% is recovered in the feces after biliary excretion. Doses should be reduced in patients with hepatic dysfunction. At least a 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

Vinblastine sulfate is available in preparations for injection. The drug is given intravenously; special precautions must be taken against subcutaneous extravasation, since this may cause painful irritation and ulceration. The drug should not be injected into an extremity with impaired circulation. After a single dose of 0.3 mg/kg of body weight, myelosuppression reaches its maximum in 7 to 10 days. If a moderate level of leukopenia (approximately 3000 cells/mm^3) is not attained, the weekly dose may be increased gradually by increments of 0.05 mg/kg of body weight. In regimens designed to cure testicular cancer, vinblastine is used in doses of 0.3 mg/kg every 3 weeks irrespective of blood cell counts or toxicity.

The most important clinical use of vinblastine is with bleomycin and cisplatin in the curative therapy of metastatic testicular tumors. Beneficial responses have been reported in various lymphomas, particularly Hodgkin's disease, where significant improvement may be noted in 50 to 90% of cases. The effectiveness of vinblastine in a high proportion of lymphomas is not diminished when the disease is refractory to alkylating agents. It is also active in Kaposi's sarcoma, neuroblastoma, and Letterer-Siwe disease (histiocytosis X), as well as in carcinoma of the breast and choriocarcinoma in women.

Doses of vinblastine will be determined by the clinician according to the individual patients need. 0.1 to 0.3mg/kg can be administered or 1.5 to 2mg/m^2 can also be administered. Alternatively, 0.1 mg/m^2, 0.12 mg/m^2, 0.14 mg/m^2, 0.15 mg/m^2, 0.2 mg/m^2, 0.25 mg/m^2, 0.5 mg/m^2, 1.0 mg/m^2, 1.2 mg/m^2, 1.4 mg/m^2, 1.5 mg/m^2, 2.0 mg/m^2, 2.5 mg/m^2, 5.0 mg/m^2, 6 mg/m^2, 8 mg/m^2, 9 mg/m^2, 10 mg/m^2, 20 mg/m^2, can be given. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

d. Vincristine

Vincristine blocks mitosis and produces metaphase arrest. It seems likely that most of the biological activities of this drug can be explained by its ability to bind specifically to tubulin and to block the ability of protein to polymerize into microtubules. Through disruption of the microtubules of the mitotic apparatus, cell division is arrested in metaphase. The inability to segregate chromosomes correctly during mitosis presumably leads to cell death.

The relatively low toxicity of vincristine for normal marrow cells and epithelial cells make this agent unusual among anti-neoplastic drugs, and it is often included in combination with other myelosuppressive agents.

Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each drug in plasma is approximately 0.4 mM.

Vinblastine and vincristine bind to plasma proteins. They are extensively concentrated in platelets and to a lesser extent in leukocytes and erythrocytes.

Vincristine has a multiphasic pattern of clearance from the plasma; the terminal half-life is about 24 hours. The drug is metabolized in the liver, but no biologically active derivatives have been identified. Doses should be reduced in patients with hepatic dysfunction. At least a 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

Vincristine sulfate is available as a solution (1 mg/ml) for intravenous injection. Vincristine used together with corticosteroids is presently the treatment of choice to induce remissions in childhood leukemia; the optimal dosages for these drugs appear to

be vincristine, intravenously, 2 mg/m^2 of body-surface area, weekly, and prednisone, orally, 40 mg/m^2, daily. Adult patients with Hodgkin's disease or non-Hodgkin's lymphomas usually receive vincristine as a part of a complex protocol. When used in the MOPP regimen, the recommended dose of vincristine is 1.4 mg/m^2. High doses of vincristine seem to be tolerated better by children with leukemia than by adults, who may experience sever neurological toxicity. Administration of the drug more frequently than every 7 days or at higher doses seems to increase the toxic manifestations without proportional improvement in the response rate. Precautions should also be used to avoid extravasation during intravenous administration of vincristine. Vincristine (and vinblastine) can be infused into the arterial blood supply of tumors in doses several times larger than those that can be administered intravenously with comparable toxicity.

Vincristine has been effective in Hodgkin's disease and other lymphomas. Although it appears to be somewhat less beneficial than vinblastine when used alone in Hodgkin's disease, when used with mechlorethamine, prednisone, and procarbazine (the so-called MOPP regimen), it is the preferred treatment for the advanced stages (III and IV) of this disease. In non-Hodgkin's lymphomas, vincristine is an important agent, particularly when used with cyclophosphamide, bleomycin, doxorubicin, and prednisone. Vincristine is more useful than vinblastine in lymphocytic leukemia. Beneficial response have been reported in patients with a variety of other neoplasms, particularly Wilms' tumor, neuroblastoma, brain tumors, rhabdomyosarcoma, and carcinomas of the breast, bladder, and the male and female reproductive systems.

Doses of vincristine for use will be determined by the clinician according to the individual patients need. 0.01 to 0.03mg/kg or 0.4 to 1.4mg/m^2 can be administered or 1.5 to 2mg/m^2 can alos be administered. Alternatively 0.02 mg/m^2, 0.05 mg/m^2, 0.06 mg/m^2, 0.07 mg/m^2, 0.08 mg/m^2, 0.1 mg/m^2, 0.12 mg/m^2, 0.14 mg/m^2, 0.15 mg/m^2, 0.2 mg/m^2, 0.25mg/m^2 can be given as a constant intravenous infusion. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

e. Camptothecin

Camptothecin is an alkaloid derived from the chinese tree *Camptotheca acuminata* Decne. Camptothecin and its derivatives are unique in their ability to inhibit DNA Topoisomerase by stabilizing a covalent reaction intermediate, termed "the cleavable complex," which ultimately causes tumor cell death. It is widely believed that camptothecin analogs exhibited remarkable anti-tumour and anti-leukaemia activity. Application of camptothecin in clinic is limited due to serious side effects and poor water-solubility. At present, some camptothecin analogs (topotecan; irinotecan), either synthetic or semi-synthetic, have been applied to cancer therapy and have shown satisfactory clinical effects. The molecular formula for camptothecin is $C_{20}H_{16}N_2O_4$, with a molecular weight of 348.36. It is provided as a yellow powder, and may be solubilized to a clear yellow solution at 50 mg/ml in DMSO 1N sodium hydroxide. It is stable for at least two years if stored at 2-8°X in a dry, airtight, light-resistant environment.

5. Nitrosureas

Nitrosureas, like alkylating agents, inhibit DNA repair proteins. They are used to treat non-Hodgkin's lymphomas, multiple myeloma, malignant melanoma, in addition to brain tumors. Examples include carmustine and lomustine.

a. Carmustine

Carmustine (sterile carmustine) is one of the nitrosoureas used in the treatment of certain neoplastic diseases. It is 1,3bis (2-chloroethyl)-1-nitrosourea. It is lyophilized pale yellow flakes or congealed mass with a molecular weight of 214.06. It is highly soluble in alcohol and lipids, and poorly soluble in water. Carmustine is administered by intravenous infusion after reconstitution as recommended. Sterile carmustine is commonly available in 100 mg single dose vials of lyophilized material.

Although it is generally agreed that carmustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins.

Carmustine is indicated as palliative therapy as a single agent or in established combination therapy with other approved chemotherapeutic agents in brain tumors such as glioblastoma, brainstem glioma, medullobladyoma, astrocytoma, ependymoma, and metastatic brain tumors. Also it has been used in combination with prednisone to treat multiple myeloma. Carmustine has proved useful, in the treatment of Hodgkin's Disease and in non-Hodgkin's lymphomas, as secondary therapy in combination with other approved drugs in patients who relapse while being treated with primary therapy, or who fail to respond to primary therapy.

The recommended dose of carmustine as a single agent in previously untreated patients is 150 to 200 mg/m^2 intravenously every 6 weeks. This may be given as a single dose or divided into daily injections such as 75 to 100 mg/m^2 on 2 successive days. When carmustine is used in combination with other myelosuppressive drugs or in patients in whom bone marrow reserve is depleted, the doses should be adjusted accordingly. Doses subsequent to the initial dose should be adjusted according to the hematologic response of the patient to the preceding dose. It is of course understood that other doses may be used in the present invention for example $10mg/m^2$, $20mg/m^2$, $30mg/m^2$ $40mg/m^2$ $50mg/m^2$ $60mg/m^2$ $70mg/m^2$ $80mg/m^2$ $90mg/m^2$ $100mg/m^2$. The skilled artisan is directed to, "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

b. Lomustine

Lomustine is one of the nitrosoureas used in the treatment of certain neoplastic diseases. It is 1-(2-chloro-ethyl)-3-cyclohexyl-1 nitrosourea. It is a yellow powder with the empirical formula of $C_9H_{16}ClN_3O_2$ and a molecular weight of 233.71. Lomustine is soluble in 10% ethanol (0.05 mg per mL) and in absolute alcohol (70 mg per mL). Lomustine is relatively insoluble in water (<0.05 mg per mL). It is relatively unionized at a physiological pH. Inactive ingredients in lomustine capsules are: magnesium stearate and mannitol.

Although it is generally agreed that lomustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins.

Lomustine may be given orally. Following oral administration of radioactive lomustine at doses ranging from 30 mg/m^2 to 100 mg/m^2, about half of the radioactivity given was excreted in the form of degradation products within 24 hours. The serum half-life of the metabolites ranges from 16 hours to 2 days. Tissue levels are comparable to plasma levels at 15 minutes after intravenous administration.

Lomustine has been shown to be useful as a single agent in addition to other treatment modalities, or in established combination therapy with other approved chemotherapeutic agents in both primary and metastatic brain tumors, in patients who have already received appropriate surgical and/or radiotherapeutic procedures. It has also proved effective in secondary therapy against Hodgkin's Disease in combination with other approved drugs in patients who relapse while being treated with primary therapy, or who fail to respond to primary therapy.

The recommended dose of lomustine in adults and children as a single agent in previously untreated patients is 130 mg/m^2 as a single oral dose every 6 weeks. In individuals with compromised bone marrow function, the dose should be reduced to 100 mg/m^2 every 6 weeks. When lomustine is used in combination with other myelosuppressive drugs, the doses should be adjusted accordingly. It is understood that other doses may be used for example, 20 mg/m^2 30 mg/m^2, 40 mg/m^2, 50 mg/m^2, 60 mg/m^2, 70 mg/m^2, 80 mg/m^2, 90 mg/m^2, 100 mg/m^2, 120 mg/m^2 or any doses between these figures as determined by the clinician to be necessary for the individual being treated.

6. Other Agents

Other agents that may be used include Avastin, Iressa, Erbitux, Velcade, and. Gleevec. In addition, growth factor inhibitors and small molecule kinase inhibitors have utility in the present invention as well. All therapies described in Cancer: Principles and Practice of Oncology Single Volume (Book with CD-ROM) by Vincent T. Devita (Editor), Samuel Hellman (Editor), Steven A. Rosenberg (Editor) Lippencott (2001), are

hereby incorporated by reference. The following additional therapies are encompassed, as well.

a. Immunotherapy

Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

Immunotherapy, thus, could be used as part of a combined therapy, in conjunction with Ad-mda7 gene therapy. The general approach for combined therapy is discussed below. Generally, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, *erb* B and p155.

Tumor Necrosis Factor is a glycoprotein that kills some kinds of cancer cells, activates cytokine production, activates macrophages and endothelial cells, promotes the production of collagen and collagenases, is an inflammatory mediator and also a mediator of septic shock, and promotes catabolism, fever and sleep. Some infectious agents cause tumor regression through the stimulation of TNF production. TNF can be quite toxic when used alone in effective doses, so that the optimal regimens probably will use it in lower doses in combination with other drugs. Its immunosuppressive actions are potentiated by gamma-interferon, so that the combination potentially is dangerous. A hybrid of TNF and interferon-α also has been found to possess anti-cancer activity.

b. Hormonal Therapy

The use of sex hormones according to the methods described herein in the treatment of cancer. While the methods described herein are not limited to the treatment of a specific cancer, this use of hormones has benefits with respect to cancers of the breast, prostate, and endometrial (lining of the uterus). Examples of these hormones are estrogens, anti-estrogens, progesterones, and androgens.

Corticosteroid hormones are useful in treating some types of cancer (lymphoma, leukemias, and multiple myeloma). Corticosteroid hormones can increase the effectiveness of other chemotherapy agents, and consequently, they are frequently used in combination treatments. Prednisone and dexamethasone are examples of corticosteroid hormones.

D. Radiotherapy

Radiotherapy, also called radiation therapy, is the treatment of cancer and other diseases with ionizing radiation. Ionizing radiation deposits energy that injures or destroys cells in the area being treated by damaging their genetic material, making it impossible for these cells to continue to grow. Although radiation damages both cancer cells and normal cells, the latter are able to repair themselves and function properly. Radiotherapy may be used to treat localized solid tumors, such as cancers of the skin, tongue, larynx, brain, breast, or cervix. It can also be used to treat leukemia and lymphoma (cancers of the blood-forming cells and lymphatic system, respectively).

Radiation therapy used according to the present invention may include, but is not limited to, the use of γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

Radiotherapy may comprise the use of radiolabeled antibodies to deliver doses of radiation directly to the cancer site (radioimmunotherapy). Antibodies are highly specific proteins that are made by the body in response to the presence of antigens (substances recognized as foreign by the immune system). Some tumor cells contain specific antigens that trigger the production of tumor-specific antibodies. Large quantities of these antibodies can be made in the laboratory and attached to radioactive substances (a process known as radiolabeling). Once injected into the body, the antibodies actively seek out the cancer cells, which are destroyed by the cell-killing (cytotoxic) action of the radiation. This approach can minimize the risk of radiation damage to healthy cells.

Conformal radiotherapy uses the same radiotherapy machine, a linear accelerator, as the normal radiotherapy treatment but metal blocks are placed in the path of the x-ray beam to alter its shape to match that of the cancer. This ensures that a higher radiation dose is given to the tumor. Healthy surrounding cells and nearby structures receive a lower dose of radiation, so the possibility of side effects is reduced. A device called a multi-leaf collimator has been developed and can be used as an alternative to the metal blocks. The multi-leaf collimator consists of a number of metal sheets which are fixed to the linear accelerator. Each layer can be adjusted so that the radiotherapy beams can be shaped to the treatment area without the need for metal blocks. Precise positioning of the radiotherapy machine is very important for conformal radiotherapy treatment and a special scanning machine may be used to check the position of your internal organs at the beginning of each treatment.

High-resolution intensity modulated radiotherapy also uses a multi-leaf collimator. During this treatment the layers of the multi-leaf collimator are moved while the treatment is being given. This method is likely to achieve even more precise shaping of the treatment beams and allows the dose of radiotherapy to be constant over the whole treatment area.

Although research studies have shown that conformal radiotherapy and intensity modulated radiotherapy may reduce the side effects of radiotherapy treatment, it is possible that shaping the treatment area so precisely could stop microscopic cancer cells just outside the treatment area being destroyed. This means that the risk of the cancer coming back in the future may be higher with these specialized radiotherapy techniques.

Stereotactic radiotherapy is used to treat brain tumours. This technique directs the radiotherapy from many different angles so that the dose going to the tumour is very high and the dose affecting surrounding healthy tissue is very low. Before treatment, several scans are analysed by computers to ensure that the radiotherapy is precisely targeted, and the patient's head is held still in a specially made frame while receiving radiotherapy. Several doses are given.

Stereotactic radio-surgery (gamma knife) for brain tumors does not use a knife, but very precisely targeted beams of gamma radiotherapy from hundreds of different angles. Only one session of radiotherapy, taking about four to five hours, is needed. For this treatment you will have a specially made metal frame attached to your head. Then several scans and x-rays are carried out to find the precise area where the treatment is needed. During the radiotherapy, the patient lies with their head in a large helmet, which has hundreds of holes in it to allow the radiotherapy beams through.

Scientists also are looking for ways to increase the effectiveness of radiation therapy. Two types of investigational drugs are being studied for their effect on cells undergoing radiation. Radiosensitizers make the tumor cells more likely to be damaged, and radioprotectors protect normal tissues from the effects of radiation. Hyperthermia, the use of heat, is also being studied for its effectiveness in sensitizing tissue to radiation.

VII. Other Therapeutic Combinations

In accordance with the present invention, additional therapies may be applied with further benefit to the patients. Such therapies include surgery, cytokines, toxins, drugs, dietary, or a non-p53-based gene therapy. Examples are discussed below.

A. Subsequent Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

Upon excision of part of all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

B. Gene Therapy

In another embodiment, the secondary treatment is a non-p53 gene therapy in which a second gene is administered to the subject. Delivery of a vector encoding p53 in conjuction with a second vector encoding one of the following gene products may be utilized. Alternatively, a single vector encoding both genes may be used. A variety of moleclues are encompassed within this embodiment, some of which are described below.

1. Inducers of Cellular Proliferation

The proteins that induce cellular proliferation further fall into various categories dependent on function. The commonality of all of these proteins is their ability to regulate cellular proliferation. For example, a form of PDGF, the sis oncogene, is a secreted growth factor. Oncogenes rarely arise from genes encoding growth factors, and at the present, sis is the only known naturally-occurring oncogenic growth factor. In one embodiment of the present invention, it is contemplated that anti-sense mRNA directed to a particular inducer of cellular proliferation is used to prevent expression of the inducer of cellular proliferation.

The proteins FMS, ErbA, ErbB and neu are growth factor receptors. Mutations to these receptors result in loss of regulatable function. For example, a point mutation affecting the transmembrane domain of the Neu receptor protein results in the neu oncogene. The erbA oncogene is derived from the intracellular receptor for thyroid hormone. The modified oncogenic ErbA receptor is believed to compete with the endogenous thyroid hormone receptor, causing uncontrolled growth.

The largest class of oncogenes includes the signal transducing proteins (*e.g.*, Src, Abl and Ras). The protein Src is a cytoplasmic protein-tyrosine kinase, and its transformation from proto-oncogene to oncogene in some cases, results via mutations at tyrosine residue 527. In contrast, transformation of GTPase protein ras from proto-oncogene to oncogene, in one example, results from a valine to glycine mutation at amino acid 12 in the sequence, reducing ras GTPase activity.

The proteins Jun, Fos and Myc are proteins that directly exert their effects on nuclear functions as transcription factors.

2. Inhibitors of Cellular Proliferation

The tumor suppressor oncogenes function to inhibit excessive cellular proliferation. The inactivation of these genes destroys their inhibitory activity, resulting in unregulated proliferation. The tumor suppressors Rb, p16, MDA-7, PTEN and C-CAM are specifically contemplated.

3. Regulators of Programmed Cell Death

Apoptosis, or programmed cell death, is an essential process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Cleary *et al.*, 1986; Tsujimoto *et al.*, 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2

protein now is recognized to be a member of a family of related proteins, which can be categorized as death agonists or death antagonists.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (*e.g.*, Bcl_{XL}, Bcl_{W}, Bcl_{S}, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (*e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

VIII. Pharmaceutical Compositions

According to the present invention, therapeutic compositions are administered to a subject. The phrases "pharmaceutically" or "pharmacologically acceptable" refer to compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the compositions, vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

In various embodiments, agents that might be delivered may be formulated and administered in any pharmacologically acceptable vehicle, such as parenteral, topical, aerosal, liposomal, nasal or ophthalmic preparations. In certain embodiments, formulations may be designed for oral, inhalant or topical administration. In those situations, it would be clear to one of ordinary skill in the art the types of diluents that would be proper for the proposed use of the polypeptides and any secondary agents required.

Administration of compositions according to the present invention will be via any common route so long as the target tissue or surface is available via that route. This includes oral, nasal, buccal, respiratory, rectal, vaginal or topical. Alternatively,

administration may be by intratumoral, intralesional, into tumor vasculature, local to a tumor, regional to a tumor, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection (systemic). Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial an antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile

vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. Routes of administration may be selected from intravenous, intrarterial, intrabuccal, intraperitoneal, intramuscular, subcutaneous, oral, topical, rectal, vaginal, nasal and intraocular.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill

in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

In a particular embodiment, liposomal formulations are contemplated. Liposomal encapsulation of pharmaceutical agents prolongs their half-lives when compared to conventional drug delivery systems. Because larger quantities can be protectively packaged, this allows the opportunity for dose-intensity of agents so delivered to cells.

IX. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: MATERIALS AND METHODS

Three open label Phase 2 clinical trials were conducted to examine the efficacy of adenoviral-p53 (Advexin®) therapy on recurrent squamous cancer cell of the head and neck (SCCHN). Qualifications for the trails were local or regional recurrent SCCHN, prior treatment with standard radiation (5000 cGy), bidimensionally measurable disease (7.5 cm), absence of CNS metastasis, and Karnofsky performance status of ≥ 60%. Several different treatment regimens were included:

<u>T207 (high dose)</u>: 0.5 – 2 x 10^{12} viral particles, based on tumor volume (regimen A = injection on day 1; regimen B = injection on days 1, 3, 5, 8, 10 and 12);

<u>T201 (high dose)</u>: 0.5 – 2 x 10^{12} viral particles, based on tumor volume (regimen A = injection on days 1, 2 and 3; regimen B = injection on days 1, 3, 5, 8, 10 and 12); and

<u>T202 (high dose)</u>: 0.1 – 4 x 10^{10} viral particles, based on tumor volume (injection on days 1, 2 and 3).

Injections were intratumoral. As stated above, all patients had been treated with prior radiation therapy and 59% had previous chemotherapy.

<u>EXAMPLE 2: RESULTS</u>

The objective of these studies was to evaluate the safety and efficacy of adenoviral p53 gene therapy. The objective overall response rate of ADVEXIN monotherapy was 10% (complete and partial response with > 50% reduction in tumor size). Tumor growth control (stable disease or better) was achieved in 59% of all treated lesions. FIG. 1. An ADVEXIN dose response was observed in patients who received at least one cycle of treatment and patients treated with higher doses had a statistically significant increase in median survival (T201 + T207 vs. T202, 243 vs. 119 days, p=0.0096). FIGS. 2-3.

The overall median survival was longer than expected in patients who were treated with ADVEXIN® followed by chemotherapy in each of the studies: T202 (n=20) 330 days; T201 (n=47) 260 days; T207 (n=29) 246 days. The chemotherapy regimens combined with ADVEXIN® contained standard agents commonly administered to patients with recurrent disease: platinum (67%), taxanes (35%), methotexate (31%), 5-FU (27%) and bleomycin (8%). A longer than expected median survival was observed in patients with recurrent, re-treated disease (n=75) who received the higher dose of ADVEXIN®: 209 vs. 105 days, p=0.0163. There were no significant differences between the treatment groups in prior chemotherapy, time from diagnosis, Karnofsky status or sites or size of tumors.

ADVEXIN® treatment-related side effects were generally mild to moderate in nature and included transient injection site pain and fever. In conclusion, the results from

these three independent Phase II studies indicate that intratumoral injection with ADVEXIN® in patients with recurrent SCCHN caused a 10% objective response and 59% tumor growth control. Moreover, treatment with ADVEXIN® in combination with subsequent chemotherapy in previously treated patients with recurrent SCCHN resulted in longer than expected median survival.

EXAMPLE 3 – PATIENT PROFILES

Patient 10309 (Study T201) was diagnosed with a Stage IV squamous cell cancer of the head and neck in August, 1997. On August 22, the patient underwent a radical neck dissection, which was followed by full dose radiation treatment (September 26 – October 11, 1997). In March of 1998, the tumor recurred (two lesions) and the patient was entered into Study T201. The patient was randomized to receive 3 intratumoral injections into each of the recurrences every treatment cycle for up to 6 cycles. Due to disease progression, the patient was taken off the study on June 8, 1998 after two cycles of treatment (during March and April). On June 9 and on September 9 the patient was treated with docetaxel and carboplatin (two cycles 3 months apart). No other tumor therapy was administered and the patient expired on February 2, 1999 (survival 331 days since entry into Study T201). The survival was longer than expected.

Patient 50907 (Study T201) was diagnosed with squamous cell cancer of the head and neck in April, 1988. Between 1988 and 1998 the patient went through several surgeries due to disease progression. Full dose radiation was given from February though April and July through September, 1993 (complete response). Before being entered into Study T201, the patient was treated with the following anti-tumor treatment: 13 cis-retinoic acid (1994 – 1996), α-interferon (1996), methotrexate (1996 - 1997), leucovorin (1998) and methotrexate (1998). During the last methotrexate treatment, the disease progressed. The patient was randomized into Study T201 on December 17, 1998. The patient was randomized to receive three intra-tumoral injections of Advexin® per cycle. One lesion was to be treated. After two cycles of treatment the patient was removed from the study treatment due to progressive disease (2/25/99). On March 9, 1999, the patient received one cycle of Taxol in combination with carboplatin, ifosfamide and Mesna. The

patient expired on December 2, 1999 (340 day survival) The survival was longer than expected.

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All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

X. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

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U.S. Patent 4,659,774
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U.S. Patent 4,682,195
U.S. Patent 4,683,202
U.S. Patent 4,683,202
U.S. Patent 4,797,368
U.S. Patent 4,797,368
U.S. Patent 4,816,571
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U.S. Patent 4,879,236
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U.S. Patent 4,959,463
U.S. Patent 4,959,463
U.S. Patent 5,139,941
U.S. Patent 5,139,941
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U.S. Patent 5,538,880

U.S. Patent 5,550,318

U.S. Patent 5,550,318

U.S. Patent 5,554,744

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U.S. Patent 5,563,055

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U.S. Patent 5,702,932

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U.S. Patent 5,736,524

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U.S. Patent 5,747,469

U.S. Patent 5,747,469

U.S. Patent 5,747,869

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U.S. Patent 5,780,448

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U.S. Patent 5,789,215

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U.S. Patent 5,871,986

U.S. Patent 5,871,986

U.S. Patent 5,879,703

U.S. Patent 5,879,703

U.S. Patent 5,932,210

U.S. Patent 5,932,210

U.S. Patent 5,945,100

U.S. Patent 5,945,100

U.S. Patent 5,981,225

U.S. Patent 5,981,225

U.S. Patent 5,981,274

U.S. Patent 5,981,274

U.S. Patent 5,994,136

U.S. Patent 5,994,624

U.S. Patent 5,994,624

U.S. Patent 6,013,516

U.S. Patent 6,013,516

U.S. Patent 6,017,524

U.S. Patent 6,017,524

U.S. Patent 6,069,134

U.S. Patent 6,069,134

U.S. Patent 6,136,594

U.S. Patent 6,136,594

U.S. Patent 6,143,290

U.S. Patent 6,143,290

U.S. Patent 6,143,290

U.S. Patent 6,143,290

U.S. Patent 6,210,939

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U.S. Patent 6,296,845

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